Expert Opinion

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Targeted delivery across the blood–brain barrier

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The safest and most effective way of targeting drugs to the entire brain is via delivery systems directed at endogenous receptor-mediated uptake mechanisms present at the cerebral capillaries. Such systems have been shown to be effective in animal models including primates, but no clinical trials have been performed so far. This review focuses on the well-characterised transferrin and insulin receptor-targeted systems, as well as on the more recently described systems that use the low-density lipoprotein-related protein 1 receptor, the low-density lipoprotein-related protein 2 receptor (also known as megalin and glycoprotein 330) or the diphtheria toxin receptor (which is the membrane-bound precursor of heparin-binding epidermal growth factor-like growth factor). The possibilities and limitations of these systems are compared and their future for human application is discussed.

Keywords: blood–brain barrier, CNS, diphtheria toxin receptor, drug targeting, insulin receptor, LRP1 receptor, LRP2 receptor, receptor-mediated endocytosis, transferrin receptor

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1. Introduction

The CNS is protected by the blood–brain barrier. This barrier regulates the transport of endogenous and exogenous compounds by controlling their selective and specific uptake, efflux and metabolism into and out of the brain. Unfortunately, potential drugs for the treatment of most brain diseases are, therefore, often also not able to cross the blood–brain barrier. As a result, various drug delivery and targeting strategies are currently being developed. This review discusses the biology and physiology of the blood–brain barrier, with a focus on receptor-mediated drug delivery to the (human) brain.

2. The blood–brain barrier

The first evidence for the existence of a barrier between blood and brain was discovered by Ehrlich (1885), who injected the dye Trypan blue intravenously and found that, in contrast to other tissues, it did not stain the brain [1]. In a second series of experiments Goldman (1913) injected the dye into the cerebral spinal fluid, after which staining of the brain was observed but not of the peripheral organs [2]. Since this seminal discovery, much research has been performed on the pathophysiology and pharmacology of the blood–brain barrier.

The current knowledge is that the blood–brain barrier is situated at the interface between the blood and the brain and its primary function is to maintain the homeostasis of the brain. In addition to the blood–brain barrier, there is a second barrier at the blood–cerebrospinal fluid (CSF) interface, presented by the choroid plexus epithelium [3]. Furthermore, the blood–brain barrier is not uniform throughout the brain, as the capillaries in the circumventricular organs (CVOs) are fenestrated [4,5]. **Figure 1** gives a schematic representation of the barriers present in the CNS.

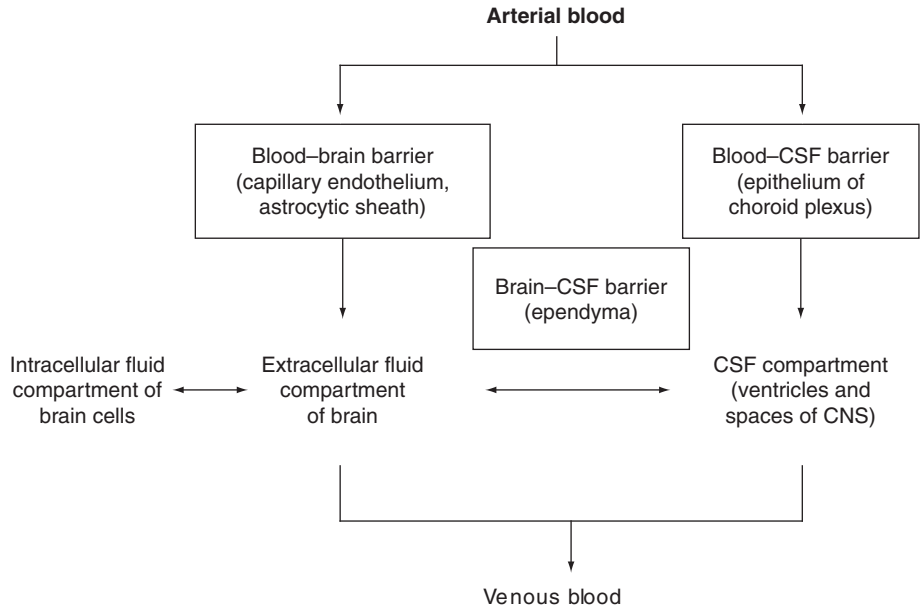

Figure 1. Schematic representation of the blood–brain barrier, the blood–CSF barrier and the brain–CSF barrier. The blood–brain barrier has the largest surface area, and is, therefore, considered to be the most important influx barrier for solutes directed to the brain.
CSF: Cerebrospinal fluid.

The human blood–brain barrier has a total blood vessel length of ~ 600 km and an estimated surface area of ~ 20 m^2, which makes it significantly larger than the blood–CSF or the brain–CSF barrier [6]. Therefore, the blood–brain barrier is considered to be the most important influx barrier for solutes directed to the brain [4,5].

The blood–brain barrier is mainly formed by brain capillary endothelial cells (BCECs) [7], although other cell types such as pericytes, astrocytes and neuronal cells also play an important role in its function [8]. BCECs are different from peripheral endothelial cells (**Figure 2**). BCECs have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water soluble) compounds from blood to the brain [7,9,10]. Furthermore, transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity and a lack of fenestrae [8]. These specific characteristics of the blood–brain barrier are induced and maintained by the endfeet of astrocytes surrounding the BCECs [7,11] and by neuronal endings, which can directly innervate the BCECs [8,12]. Pericytes also play a role at the blood–brain barrier, as they share the continuous capillary basement membrane with the BCECs. Pericytes cover ~ 20 – 30% of the cerebral capillary surface, so they do not really constitute a physical barrier for the movement of solutes across the BCECs; however, their phagocytotic activity does form an additional blood–brain barrier property [5,13]. Because of these complex interactions between cell types, as well as the dynamic regulation of the blood–brain barrier properties (e.g., receptor expression, formation of tight junctions), the blood–brain barrier is considered to be an organ protecting the brain [14].

The function of the blood–brain barrier is to exclude potential neurotoxic endogenous and exogenous compounds from the brain, to maintain ion homeostasis and to nourish the brain with essential nutrients such as glucose, amino acids, purines, nucleosides, peptides and proteins [15,16]. Several influx mechanisms exist, which can be divided into active or passive blood–brain barrier transport mechanisms. Passive diffusion depends on lipophilicity and molecular weight [17]. Furthermore, the ability of a compound to form hydrogen bonds will limit its diffusion through the blood–brain barrier [18]. In general, Lipinski's rule of five, and the Abraham's equation can be used to predict the passive transport of a drug molecule across the blood–brain barrier [19,20]. Transport of hydrophilic compounds via the paracellular route is limited, whereas lipophilic drugs of < 400 – 600 Da may freely enter the brain via the transcellular route. Facilitated transport systems can be divided into absorptive-, carrier- or receptor-mediated transcytosis.

Absorptive-mediated transcytosis is initiated by the binding of polycationic substances (e.g., most cell-penetrating peptides) to negative charges on the plasma membrane [21,22]. This process does not involve specific plasma membrane receptors. Following the binding of the cationic compound to the plasma membrane, endocytosis occurs, followed by the formation of endosomes. Indeed, several drugs have been described to enter the brain via this mechanism [23,24]. However, vesicular transport is actively downregulated in the blood–brain barrier to protect the brain from non-specific exposure to polycationic compounds. Therefore, forcing drugs to enter the brain by absorptive-mediated transcytosis

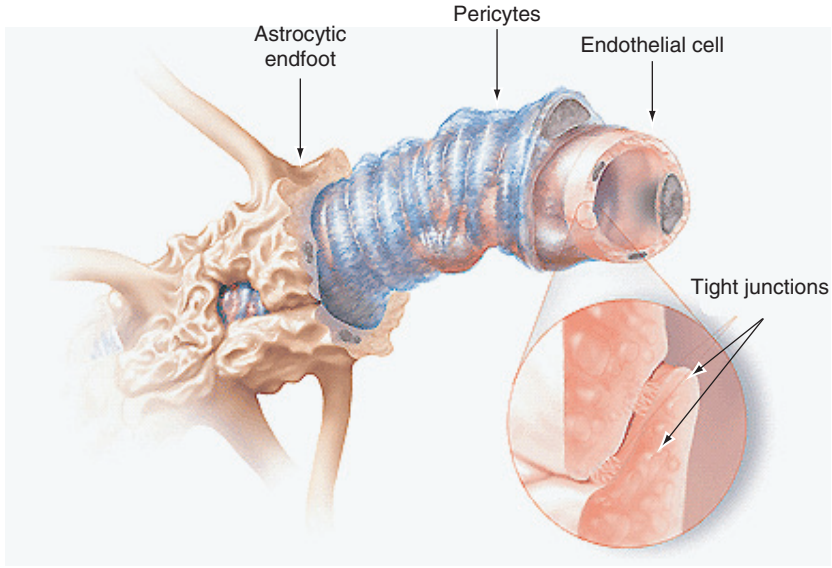

Figure 2. Schematic representation of a cerebral capillary. Note the surrounding pericytes (covering ~ 20 – 30% of the capillary surface) and astrocytic endfeet projecting on to the endothelial cells of the cerebral capillaries that induce and maintain the blood–brain barrier. In contrast, endothelial cells of peripheral capillaries do not form a tight barrier because they lack the specific input of these brain cells. Reprinted with permission from MILLER G: Drug targeting. Breaking down barriers. *Science* (2002) **297**(5584):1116-1118, © 2002 American Association for the Advancement of Science. Illustration: C Slayden.

may go against the neuroprotective barrier function, as was recently illustrated for anionic and cationic nanoparticles that were shown to disrupt the blood–brain barrier [25].

Carrier-mediated transcytosis is used for the transcytosis of nutrients, such as glucose, amino acids and purine bases [26-28]. At least eight different nutrient transport systems have been identified, which each transport a group of nutrients of the same structure. Carrier-mediated transcytosis is substrate selective and the transport rate is dependent on the degree of occupation of the carrier [28]. Therefore, only drugs that closely mimic the endogenous carrier substrates will be taken up and transported into the brain.

The focus of this review will be on receptor-mediated transcytosis, which enables larger molecules, such as peptides, proteins and DNA, to specifically enter the brain. Classical examples of receptors involved in receptor-mediated transcytosis are the insulin receptor [29], the transferrin receptor [30,31] and the transporters for low-density lipoprotein [32], leptin [33] and insulin-like growth factors [34].

Besides many influx mechanisms, several efflux mechanisms also exist at the blood–brain barrier. The best known is P-glycoprotein (P-gp). P-gp is a transmembrane protein commonly thought to be located at the apical membrane of the BCECs, although some recent publications describe conditional P-gp expression on astrocytes and astrocyte endfeet as well [35]. It has a high affinity for a wide range of compounds, including cytotoxic anticancer drugs, antibiotics, hormones and HIV protease inhibitors [36]. Other multi-drug resistance (MDR) efflux mechanisms at the blood–brain barrier include the MDR-related proteins (MRPs), such as MRP1, 2, 5 and 6 [37]. In addition, many other transporters are present at the blood–brain barrier, such as the organic anion transporter (influx and efflux), the organic cation transport system (influx) and the nucleoside transporter system (influx) [14,38].

In conclusion, research over the years has shown that the blood–brain barrier is a dynamic organ, which combines restricted diffusion to the brain for endogenous and exogenous compounds, with specialised transport mechanisms for essential nutrients.

3. Drug delivery and targeting strategies to the brain

For many diseases of the brain, such as Alzheimer's disease, Parkinson's disease, stroke, depression, schizophrenia, epilepsy and migraine headache, the drugs on the market are far from ideal, and none are curative. A significant part of the problem is the poor blood–brain barrier penetration of most of the drugs in development against neuronal targets for treatment of these disorders. This includes ~ 98% of the small molecules and nearly 100% of large molecules, such as recombinant proteins or gene-based medicines [39]. Therefore, much effort is directed towards delivery and targeting of drugs to the brain. Drug delivery to the brain can be achieved via several methods, including invasive, pharmacochemical or physiological strategies.

Invasive brain drug delivery strategies, such as direct intracerebral injections of slow-release products, for example, only allow for local delivery (**Figure 3B**). This may be effective for drug delivery to localised brain tumours, but not for the

administration of drugs against more widespread diseases. Another invasive method is intracerebroventricular or intrathecal drug infusion, in which a drug is directly injected into the CSF. However, CSF is completely drained into the venous circulation, and drugs still have to cross the ependymal brain–CSF barrier. As a result, the infused drug has minimal access to the parenchyma by diffusion (**Figure 3C**) [40]. In general, invasive strategies are not effective for drug delivery to the whole brain, but only to a localised part of the brain.

The advantage of the vascular route is the widespread diffusion of the infused drug across the whole brain [3]. This can be explained by the large surface area of the human blood–brain barrier (~ 20 m^2). In addition, it is estimated that each neuron has its own brain capillary for oxygen supply, as well as the supply of other nutrients (**Figure 3A**). In fact, every cm^3 of cortex comprises the sum of 1 km of blood vessel. This means that the vascular route is a very promising one for drug targeting and delivery to the brain. Drug delivery through blood–brain barrier disruption by osmotic imbalance or vaso-active compounds, although effective in reaching the entire brain, has the disadvantage that neurons may be damaged permanently due to unwanted blood components entering the brain [41]. In contrast, physiological drug delivery strategies aim to use endogenous transport mechanisms such as carrier- or receptor-mediated transcytosis. The focus of this review is on receptor-mediated transcytosis, therefore, several examples of receptor-mediated transcytosis that have successfully been employed to target drugs to the brain will be discussed in Section 4.

4. Receptor-mediated drug delivery to the brain

In general, receptor-mediated transcytosis occurs in three steps: receptor-mediated endocytosis of the compound at the luminal (blood) side, movement through the endothelial cytoplasm and exocytosis of the drug at the abluminal (brain) side of the brain capillary endothelium [5]. Following receptor–ligand internalisation, chlathrin-coated vesicles are formed, which are ~ 120 nm in diameter [42,43]. Therefore, receptor-mediated transcytosis allows the specific delivery of larger drug molecules or drug-carrying particles (such as liposomes or nanoparticles) to the brain.

4.1 The transferrin receptor

The most widely characterised receptor-mediated transcytosis system for the targeting of drugs to the brain is the transferrin receptor (TfR). The TfR is a transmembrane glycoprotein consisting of two 90-kDa subunits. A disulfide bridge links these subunits and each can bind one transferrin molecule [30]. The TfR is mainly expressed on hepatocytes, erythrocytes, intestinal cells, monocytes and on endothelial cells of the blood–brain barrier [44,45]. Furthermore, in the brain the TfR is expressed on choroid plexus epithelial cells and neurons [30]. The TfR mediates cellular uptake of iron bound to transferrin.

Drug targeting to the TfR can be by achieved either by using the endogenous ligand, transferrin, or by using an antibody directed against the TfR (OX-26; anti-rat TfR). Each of these targeting vectors has its advantages and disadvantages. For transferrin, the *in vivo* application is limited due to high endogenous concentrations of transferrin in plasma and the likely overdosing with iron when one tries to displace the endogenous transferrin with exogenously applied transferrin-containing systems. However, recent studies have shown that liposomes tagged with transferrin are suitable for drug delivery to blood–brain barrier endothelial cells *in vitro,* even in the presence of serum. OX-26 does not bind to the transferrin-binding site and is, therefore, not displaced by endogenous transferrin.

The TfR is responsible for iron transport to the brain. So far, the intracellular trafficking of transferrin and OX-26 on internalisation via the TfR has not been elucidated. Some literature reports suggest transcytosis of transferrin across the BCECs, whereas others claim endocytosis of transferrin, followed by an intracellular release of iron and a subsequent return of apo-transferrin to the apical side of the BCECs [31,46,47]. Moos and Morgan have shown that the transcytosis of iron exceeds the transcytosis of transferrin across the blood–brain barrier, supporting the second theory [30]. Furthermore, these authors have proposed a new theory in which the TfR–transferrin complex is transcytosed to the basolateral side of the BCECs, where transferrin remains bound to the TfR but iron is released into the brain extracellular fluid [48]. Subsequently, apo-transferrin bound to the TfR will recycle back to the apical side of the blood–brain barrier. This theory is supported by data from Zhang and Pardridge who found a 3.5-fold faster efflux from brain to blood of apo-transferrin than holo-transferrin [46]. In addition, in a recent publication Deane *et al.* illustrated that free iron is rapidly taken up by brain capillaries and subsequently released into the brain extracellular fluid and CSF at controlled moderate-to-slow rates [49].

The mechanism of transcytosis of OX-26 is not fully elucidated. Pardridge and colleagues have shown efficient drug targeting and delivery to the brain *in vivo* by applying OX-26 [31,50-52]. In contrast, Broadwell *et al.* have shown that both transferrin and OX-26 are able to cross the blood–brain barrier but that the transcytosis of transferrin is more efficient [53]. Furthermore, Moos and Morgan have shown that OX-26 mainly accumulates in the BCECs and not in the postcapillary compartment [54]. In addition, iron deficiency did not increase OX-26 uptake in rats. It has been demonstrated that iron deficiency causes an increase in TfR expression [44,55,56]. Therefore, it is expected that the uptake of OX-26 would also increase. The data by Moos and Morgan suggest that OX-26 transcytosis may result from a high-affinity accumulation by the BCECs, followed by a non-specific exocytosis at the basolateral side of the BCECs [54]. In addition, these authors found a periventricular localisation of OX-26, which suggests that OX-26 is probably also transported across the blood–CSF barrier.

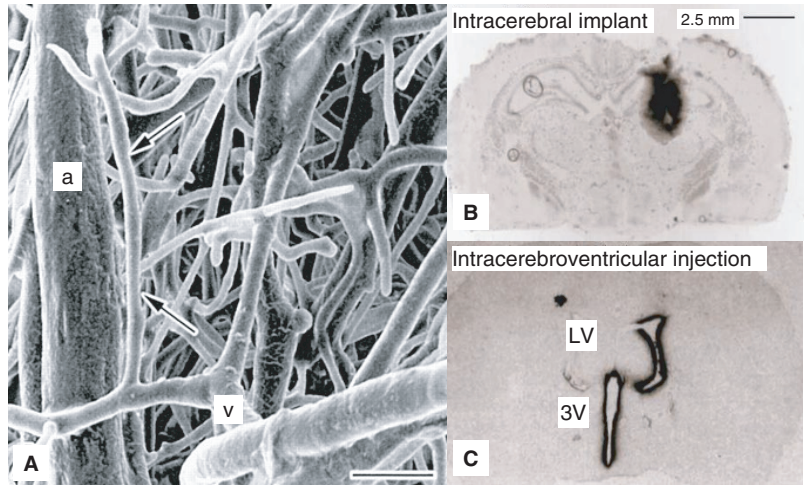

Figure 3. Drug delivery via the vascular route will enable widespread distribution of the drug to each single neuron within the brain (note that the bar in panel A indicates a length of 25 µm, which is about the size of a single neuron). A. Scanning electron micrograph of a vascular cast of a mouse brain. Reprinted from SATOMI J, MOUNT RJ, TOPORSIAN M *et al.*: Cerebral vascular abnormalities in a murine model of hereditary hemorrhagic telangiectasia. *Stroke* (2003) **34**(3):783-789, © 2003, with permission from Lippincott Williams & Wilkins. **B.** Minimal diffusion of [^{125}I]-NGF after intracerebral implantation of a biodegradable polymer (note that the bar indicates a length of 2.5 mm, which was also the size of the implant). Reprinted from KREWSON CE, KLARMAN ML, SALTZMAN WM: Distribution of nerve growth factor following direct delivery to brain interstitium. *Brain Res.* (1995) **680**(1-2):196-206, © 1995 with permission from Elsevier. **C.** Intracerebroventricular injection of [^{125}I]- BDNF. Reprinted from YAN Q, MATHESON C, SUN J, RADEKE MJ, FEINSTEIN SC, MILLER JA: Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression *Exp. Neurol.* (1994) **127**(1):23-36, © 1994 with permission from Elsevier. Note that the neurotrophin does not distribute into the brain beyond the ipsilateral ependymal surface.
3V: Third ventricle; a: Artery; BDNF: Brain-derived neurotrophic factor; LV: Left ventricle; NGF: Nerve-growth factor; v: Vein.

Although the mechanism of transcytosis of transferrin and OX-26 may not be fully elucidated, it is important to realise that drug delivery to the brain via the TfR is efficient. By these means, vasoactive intestinal peptide (VIP), brain derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), peptide nucleic acids, pegylated immunoliposomes containing plasmid DNA encoding for β-galactosidase, tyrosine hydroxylase and short hairpin RNAs, have all been made available to the brain [57]. However, OX-26 is an antibody against the rat TfR and does not bind to the human TfR, making it impossible to translate this technology to the clinic. Moreover, rat antibodies will cause immunogenic reactions in humans unless they are humanised. The preparation of humanised or chimeric antibodies is difficult and in some cases this may lead to a loss of affinity for the target receptor [58]. In addition, one can argue that the administration of antibodies directed against such an important uptake mechanism involved in iron homeostasis poses a risk for human application.

A targeting vector directed to the TfR would be small, non-immunogenic and should initialise internalisation of the TfR on binding. Xu *et al.* have used a single-chain antibody Fv fragment against the human TfR, which was tagged with a lipid anchor, for insertion into a liposomal bilayer [59]. The molecular weight of this antibody fragment, including the lipid anchor was ~ 30 kDa. In addition, Lee *et al.* (2001) have used a phage-display technique to find small peptide ligands for the human TfR [60]. They obtained a 7- and a 12-mer peptide that bind to a different binding site than transferrin and are internalised by the TfR. Although these small peptides can also exert immunogenic reactions in humans, they are promising ligands for drug targeting to the human TfR on the blood–brain barrier.

4.2 The insulin receptor

Another widely characterised, classical, receptor-mediated transcytosis system for the targeting of drugs to the brain is the insulin receptor. Again, just as for the TfR system, Pardridge and colleagues have predominantly documented the use of the insulin receptor for the targeted delivery of drugs to the brain.

The insulin receptor is a large 300-kDa protein and is a heterotetramer of two extracellular-α and two transmembrane-β subunits. Each β-chain posses tyrosine kinase activity in its cytosolic extension. The α- and β-subunits are coded by a single gene and are joined by disulfide bonds to form a cylindrical structure. Primarily, insulin binds to and changes the shape of the receptor to form a tunnel to allow entry of molecules such as glucose into the cells. The insulin receptor is a tyrosine kinase receptor and induces a complex cellular response by phosphorylating proteins on the tyrosine residues. The binding of a single insulin molecule into a pocket created by the two α-chains effects a conformational change in the insulin receptor so that the α-chains approximate one another

and carries out transphosphorylation on tyrosine residues. This autophosphorylation is necessary for the receptor to internalise into endosomes. The endosomal system has been shown to be a site where insulin signalling is regulated, but the degradation of endosomal insulin also occurs there. Most of the insulin is degraded but less so in endothelial cells [61], whereas the receptors are largely recycled to the cell surface. Endocytosis is not necessary for insulin action but is probably important for removing insulin from the cell, so the target cell for insulin responds in a time-limited fashion to the hormone. This endocytosis mechanism of the insulin receptor has been exploited for the targeting of drugs to the brain.

As for transferrin, the *in vivo* application of insulin as the carrier protein is limited, mainly due to the high required concentrations of insulin and the resulting lethal overdosing with insulin. Therefore, drug or gene delivery to rhesus monkeys, for instance, is performed with the murine 83-14 monoclonal antibody (mAb) that binds to the exofacial epitope on the α-subunit of the human insulin receptor. The mAb has a blood–brain barrier permeability surface area (PS) product in the primate that is ninefold greater than murine mAbs to the human TfR [62]. By using this mAb, Pardridge and colleagues have successfully made radiolabelled amyloid-β peptide$_{1\text{-}40}$ (A$\beta_{1\text{-}40}$), serving as a diagnostic probe for Alzheimer's disease, and pegylated immunoliposomes containing plasmid DNA encoding for β-galactosidase, available to the brain of primates [57].

Unfortunately, the 83-14 mAb cannot be used in humans due to immunogenic reactions to this mouse protein. However, genetically engineered, effective forms of the mAb have now been produced, which may allow for drug and gene delivery to the human brain [63]. Still, one can argue that the administration of antibodies directed against such an important mechanism involved in glucose homeostasis poses a risk for human application.

4.3 Lipoprotein-related protein 1 and lipoprotein-related protein 2 receptor

During the past few years, the lipoprotein-related protein 1 (LRP1) and LRP2 (also known as megalin or glycoprotein 330) receptors have been exploited to target drugs to the brain in a similar fashion as the transferrin and insulin receptors. Both LRP1 and 2 receptors belong to the structurally closely related cell surface low-density lipoprotein (LDL) receptor gene family. Both receptors are multifunctional, mutiligand scavenger and signalling receptors. A large number of substrates are shared between the two receptors, like lipoprotein lipase (LPL), α2-macroglobulin (α2M), receptor-associated protein (RAP), lactoferrin, tissue- and urokinase-type plasminogen activator (tPA/uPA), plasminogen activator inhibitor-1 (PAI-1) and tPA/uPA:PAI-1 complexes. More specific ligands for the LRP1 receptor are described to be, for example, melanotransferrin (or P97), thrombospondin 1 and 2, hepatic lipase, factor VIIa/tissue-factor pathway inhibitor, factor VIIIa, factor IXa, A$\beta_{1\text{-}40}$, amyloid-β precursor protein (APP), C1 inhibitor, complement C3, apolipoprotein E (apoE), pseudomonas exotoxin A, HIV-1 Tat protein, rhinovirus, matrix metalloproteinase-9 (MMP-9), MMP-13 (collagenase-3), spingolipid activator protein, pregnancy zone protein, antithrombin III, heparin cofactor II, α_1-antitrypsin, heat-shock protein 96 and platelet-derived growth factor (mainly involved in signalling) [64-68], where apoJ (or clusterin), amyloid-β bound to apoJ and apoE, aprotinin and very low density lipoprotein are more specific for the LRP2 receptor [69,70].

The group of Béliveau first reported that melanotransferrin/P97 was actively transcytosed across the blood–brain barrier and suggested that this was mediated by the LRP1 receptor [68]. Melanotransferrin is a membrane-bound transferrin homologue that can also exist in a soluble form, and is highly expressed on melanoma cells compared with normal melanocytes. Intravenously applied melanotransferrin delivers the majority of its bound iron to the liver and kidney, where only a small part is taken up by the brain [71]. After conjugation to melanotransferrin, Béliveau and colleagues were able to successfully deliver doxorubicin to brain tumours in animal studies [101,72]. This melanotransferrin-mediated drug targeting technology (now designated NeuroTrans™) is currently further developed by BioMarin Pharmaceuticals, Inc., for the delivery of enzyme replacement therapies to the brain. Interestingly, together with researchers from BioMarin, Pan *et al.* recently reported on the efficient transfer of RAP across the blood–brain barrier by means of the LRP1/2 receptors, suggesting to have found a novel means of protein-based drug delivery to the brain [73]. RAP is a 39-kDa protein that functions as a specialised endoplasmic reticulum chaperone assisting in the folding and trafficking of members of the LDL receptor family. In as yet unpublished results, Béliveau and colleagues have now filed a patent application on the use of the LRP2-specific ligand aprotinin, and more specifically on functional derivatives thereof (e.g., angio-pep1), thereby providing a non-invasive and flexible method and a carrier for transporting a compound or drug across the blood–brain barrier [102]. Aprotinin (Trasylol®) is known to be a potent inhibitor of serine proteases such as trypsin, plasmin, tissue and plasma kallikrein and is the only pharmacological treatment approved by the US FDA to reduce blood transfusion in coronary artery bypass grafting [74].

In addition to being a tumour marker protein, melanotransferrin is also associated with brain lesions in Alzheimer's disease and is a potential marker of the disorder [75]. In addition, the proposed receptor for melanotransferrin, LRP1, has been genetically linked to Alzheimer's disease and may influence APP processing and metabolism, and amyloid-β uptake by neurons through α2M (α2M is one of the amyloid-β carrier proteins next to apoE, apoJ, transthyretin and albumin, for example) [69,76]. Furthermore, a close relationship with the receptor for advanced glycation end products (RAGE), in shuttling amyloid-β across the blood–brain barrier, has been described [69,76]. In addition, the LRP2 receptor has also been

described as mediating the uptake of amyloid-β complexed to apoJ and apoE across the blood–brain barrier [70,77,78]. This complex interaction with Alzheimer's disease makes the safety of the use of the LRP1/2 receptors for the targeting of drugs to the brain difficult to predict in human application, especially when the complex signalling function of these receptors is included in the assessment (e.g., the control of permeability of the blood–brain barrier, vascular tone and the expression of MMPs [65]), as well as the fact that both of the receptors are critically involved in the coagulation–fibrinolysis system. Moreover, melanotransferrin was also reported to be directly involved in the activation of plasminogen [79], and high plasma concentrations of melanotransferrin are required to deliver drugs to the brain, resulting perhaps in dose limitations because of the high iron load in the body.

The same line of reasoning for the interactions at the level of the uptake receptors may apply to the use of RAP and (derivatives of) aprotinin. On the other hand, the latter has already been successfully applied to humans, usually without severe side effects, making the peptide derivatives potentially safe drug carriers. As for RAP, however, no results on the efficacy or capacity of the aprotinin peptides as carriers for drugs have yet been made available.

4.4 Diphtheria toxin receptor

Recently, our group has identified a novel human applicable carrier protein (CRM197) for the targeted delivery of conjugated proteins across the blood–brain barrier [80,103]. Uniquely, CRM197 has been used as a safe and effective carrier protein in human vaccines for a long time [81] and also recently as a systemically active therapeutic protein in anticancer trials [82]. This has resulted in a large body of prior knowledge on the carrier protein, including its transport receptor and mechanism of action, receptor binding domain, conjugation and manufacturing processes, and kinetic and safety profile both in animals and humans. CRM197 delivers drugs across the blood–brain barrier by the well-characterised, safe and effective mechanism called receptor-mediated transcytosis. From the literature, it was already known that CRM197 uses the membrane-bound precursor of heparin-binding epidermal growth factor-like growth factor (HB-EGF) as its transport receptor [83]. This precursor is also known as the diphtheria toxin receptor (DTR). In fact, CRM197 is a non-toxic mutant of diphtheria toxin. Membrane-bound HB-EGF is constitutively expressed on the blood–brain barrier, neurons and glial cells [84]. Moreover, HB-EGF expression is strongly upregulated on the cerebral blood vessels by, for example, ischaemic stroke and in gliomas [85,86], which may lead to a site-selective improvement of the therapeutic efficacy of the targeted drugs in the brain.

By means of the dynamic cell-culture model of the blood–brain barrier [87] the functional expression of the DTR, safety of the CRM197 carrier protein and specific transport efficacy of CRM197 carrier protein conjugates to a 40-kDa enzyme (horseradish peroxidase [HRP] serving as a 'model' protein drug) and DTR-targeted pegylated liposomes containing HRP were demonstrated. In addition, the *in vivo* proof-of-principle with this novel brain drug-targeting technology was demonstrated by the specific brain uptake of DTR-targeted HRP in guinea-pigs [80,103].

Although HB-EGF is expressed in species including human, monkey, rat and mouse with a similar tissue distribution, only rats and mice are resistant to diphtheria toxin because of an amino acid substitution in the receptor-binding domain on HB-EGF that reduces binding of diphtheria toxin to rodent HB-EGF [88]. Fortunately, a transgenic mouse conditionally expressing the human DTR was recently generated by Cha and colleagues [89], allowing specific study of the brain drug-delivery technology in mice as well.

Another known complication of the bacterial CRM197 protein is that neutralising antibodies against diphtheria toxin may develop or already be present in serum of the recipient because of earlier vaccinations, thereby reducing the efficacy of the drug delivery system. There are, however, several lines of evidence that such an immune response to CRM197 can occur, but, most importantly, it is not really a problem in the clinic, at least not for the treatment of acute indications. In fact, the clinical studies performed by Buzzi *et al.* indicate that pre-existing levels of neutralising antibodies were decreased 30 days after repeated treatment with CRM197 [82].

Overall, the DTR seems to be a human-applicable, safe and effective uptake receptor for the targeting of drugs to the brain, especially as CRM197 is already safely applied to humans (illustrating that binding to HB-EGF *per se* does not result in serious side effects), where other carrier systems involve potential safety hazards in human application. On the other hand, even though specific brain uptake of DTR-targeted enzymes was established in guinea-pigs, the technology now awaits further *in vivo* validation in terms of kinetics of brain distribution and efficacy of targeted drugs in relevant disease models of the CNS.

5. Expert opinion and conclusion

The multibillion dollar CNS drug market (US$58 billion in 2003) is rapidly growing (14% per year) and bears significant unmet medical needs. For this reason, the CNS drug market is considered the most promising for the whole drug industry. On the other hand, many biopharmaceutical drugs are not currently available to the brain because they are not able to cross the blood–brain barrier. The stubborn assumption that the breakdown of the blood–brain barrier (which occurs in the course of most CNS disorders) will eventually solve the problem has been proved to be wrong by those from the industry who have tried. Particularly, it either breaks down after the therapeutic time window, or the drug is still not able to cross the 'leaky' barrier in sufficient quantities to be effective. To give one devastating example, the brain is a sanctuary site for metastases of breast tumours normally curable by therapeutic anticancer antibodies that are not able to cross the

blood–brain barrier, making this type of therapy ineffective overall for these patients [90]. Brain drug-targeting technology may solve this problem.

Perhaps the most shocking conclusion from this review is that, to date, there are still no drugs in the clinic, let alone on the market, that employ brain drug-targeting technologies. Clearly, despite considerable efforts, small molecules have thus far not been able to solve the unmet needs. The characteristics required for sufficient brain penetration of small molecules are usually not in line with the safety requirements of the compounds. For that reason, more can be expected from the emerging field of biopharmaceutical drugs. Unlike small molecules, however, biopharmaceutical drugs are unlikely candidates for chemical modifications to enhance their permeability across the blood–brain barrier. Only invasive and harmful technologies, such as direct and local stereotactic injections, intrathecal infusions and blood–brain barrier disruption, are currently being evaluated in clinical settings. Because of the severe neurological consequences of these techniques, however, they are only allowed to be applied in selected life-threatening diseases. Moreover, these technologies are far from effective in delivering drugs throughout the whole brain.

As almost every neuron is perfused by its own capillary, the most effective way of delivering biopharmaceutical drugs is achieved by targeting to internalising transport receptors on these capillaries. In this review the possibilities and limitations of the human applicability for five of such receptors has been highlighted; the transferrin receptor, insulin receptor, LRP1 receptor, LRP2 receptor and DTR. So what will it take to get these systems into the clinic? Clearly the transferrin receptor-targeted system has been best validated in terms of the concept of targeted drug delivery to the brain, but not for the recently discovered human applicable peptide carriers. The insulin receptor-targeted system is the closest to human validation because of the successful proof-of-concept studies in primates. However, the potential safety issue of antibodies directed against insulin receptors has, to our knowledge, not yet been properly addressed. Still, the Santa Monica-based biotech company ArmaGen Technologies, Inc., is apparently developing insulin receptor-targeted biopharmaceutical drugs for the treatment and diagnosis of brain tumours, Alzheimer's disease and stroke on the basis of the patent estate built by Pardridge at the University of California. The Novato-based biotech company BioMarin Pharmaceuticals, Inc., forecasted last year that they would file for an investigational new drug application to test their NeuroTrans™ technology in the clinic in early 2005, but no such announcements have been made public since. Nevertheless, the targeted delivery of drugs to the brain through the LRP1 receptor may still face considerable difficulties in evading possible interactions with both the numerous other ligands of the receptor as well as its established signalling function. For these reasons, the use of uptake receptors that already have an exogenously applied ligand for the receptor being applied to humans, such as for the DTR (i.e., CRM197), may benefit considerably from the derived safety data coming from such other applications. In fact, as soon as beneficial effects of the CRM197 carrier protein conjugated to already marketed drugs are observed in relevant animal disease models, the preparation of an IND package is warranted for certain life-threatening CNS disorders.

In conclusion, what is essentially needed to get brain-targeted delivery systems into the clinic without further delay, is the involvement of companies with drugs already on the market that also need their drug to be made available to the brain. Combining such drugs to brain drug targeting and/or delivery technologies that already have individual regulatory approval for other indications, may provide the optimal base to obtain the clinical success of brain-targeted delivery systems that so many patients have been waiting for. Furthermore, drug delivery technologies combined with brain-targeting systems may also help to validate the many newly discovered drug targets in a much better way. We envision that a relevant drug target is first validated in animal disease models with the best possible class of lead compounds that usually do not cross the blood–brain barrier on their own, before chemical modifications are introduced to a preferred compound to make it available to the brain.

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